Molecular Genetic Studies of Forest Pathogens

FINAL REPORT

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INTRODUCTION

Background

Soil fungi in the genus *Armillaria* cause root and butt rot of woody plants throughout the United States. These species are prominent killers and decayers of deciduous or coniferous trees and shrubs in natural forest, plantation, orchard, agroforestry, and urban environments (Shaw and Kile 1991). *Armillaria* affects over 600 species of woody plants. It can negatively affect commercial timber production by causing tree mortality and reduced tree growth, and is a major problem for forest production in the United States and other timber producing areas. However, not all *Armillaria* species decrease forest productivity. *Armillaria* species are also common saprophytes or epiphytes in conifer forests of the northern Rocky Mountains (McDonald et al. 1987). In one study, about 60% of randomly inspected healthy Douglas-fir (*Pseudotsuga menziesii*) and grand fir (*Abies grandis*) supported epiphytic rhizomorphs (McDonald et al. 1987). As efficient decomposers, certain species of *Armillaria* should improve nutrient cycling on forest sites, and may also protect susceptible trees from pathogenic species (Brunh et al. 2000).

Although *Armillaria* may greatly affect sustainability and productivity of forest ecosystems, little is known about the key ecological interactions and genetic adaptations of species of this genus. One goal of this proposal is to obtain genetic markers to identify and survey individual *Armillaria* species. A correlation will be sought among marker-delineated species and populations and environmental factors (e.g., host species, habitat types) that may control the occurrence and pathogenicity of *Armillaria* spp. Such relationships will provide a basis for developing novel models for the prediction and management of root disease. Appropriate to the Biology of Plant-Microbe Association Program (51.8) of USDA-NRICGP, this is a mission-oriented study that uses established genetic marker methods at the landscape level to advance Armillaria root rot disease prediction and management. In addition, the integration of genetic markers, environmental data, and geographic mapping information will provide the foundation for other basic studies on the genetic structure, environmental adaptation, and gene flow of *Armillaria* species and populations.

Armillaria pathosystems are well suited for studying environmental effects (including host influences) on pathogen genetic structure. Unlike herbaceous crop systems that are subjected to annual disturbances, forests offer an ideal opportunity to study long-term interactions among pathogens, beneficial microbes, and their environments. The unique genetic characteristics of *Armillaria* species will facilitate our proposed studies on environmental adaptation in forest environments. For example, a single mycelial individual (genet) of *Armillaria* can occupy up to 1,500 ha and survive for 2,400 years in the northwestern U.S.A. (Anderson and Kohn 1995, Ferguson et al. 2000, Smith et al. 1992). In other situations, *Armillaria* genets can be quite diverse within even limited spatial area (McDonald 1999, Rizzo et al. 1995). The great diversity/variety of *Armillaria* life histories and population structures provides ideal experimental conditions conducive to exploring the interactions among environmental parameters and genetic structure. Furthermore, *Armillaria* markers that are associated with environmental factors and host tree species should provide a means to predict *Armillaria* occurrence and behavior in diverse forest ecosystems.

Information developed in this proposed study offers long-term potential for understanding interactions of *Armillaria* species and population genetic structure with environmental parameters. Prediction of *Armillaria* behavior based on genetic markers and environmental factors could be applied in the short term to help manage Armillaria root disease. The relationship of population genetic markers with habitat type and associated environmental factors should indicate conditions that are most favorable for disease development. If such situations could be identified, then forest management practices (e.g., species selection, thinning and harvest methods, and site preparation, etc.) might be modified to decrease disease severity. However, the greatest contribution of this proposed research is the development of knowledge needed to use population genetic markers in conjunction with environmental features at the landscape scale to better define the ecological roles of *Armillaria* species as an aggressive primary pathogen vs. beneficial decomposer (or potential biocontrol agent) in forest ecosystems (Bruhn et al. 2000).

Review

Armillaria root rot disease is a major problem in coniferous and hardwood forest management. About one-third of the land area of United States, 298 million ha, is forested: 154 million ha (52%) in the East, 92 million ha (31%) in the continental West, and 52 million ha (17%) in Alaska. About 28 million ha are currently at risk (where 25% or more tree mortality is expected over the next 15 years) due to insects and diseases (USDA Forest Service FHP 1999). The following four groups can account for more than 19 million ha at risk: 1) gypsy moth in the East, 2) root diseases (mainly Armillaria root rot) in the interior West, 3) southern pine beetle in the South, and 4) bark beetle in the West (USDA Forest Service FHP 1999).

Armillaria root disease occurs extensively and severely in the region of the proposed study in northern Idaho and Oregon, Washington, western Montana, and the southern interior of British Columbia. Smith (1984) estimated that 18% of the total mortality in the western North American forests is due to root disease. Annual timber losses to Armillaria root disease have been estimated at two to three million cubic meters in the western North America (Morrison and Mallett 1996).

The establishment of *Armillaria* infection centers has widespread and long-term impacts. In North Central U.S.A., a single genet (genetic individual or vegetative clone) of *A. gallica* can occupy up to 15 ha and can dominate a site for more than 1,500 years; in northwestern U.S.A., preliminary evidence shows that a genet of *A. ostoyae* can occupy up to 1,134 ha and survive for 2,400 years (Ferguson et al. 2000, Smith et al. 1992). In Douglas-fir plantations, the rate of spread of *A. ostoyae* is about 0.7 to 1.3 m/year (Peet et al. 1996). Pathogenic *Armillaria* spp. commonly kill an average of 2-4 % of susceptible trees per year, and can culminate in the removal of most susceptible trees by 80-100 years (Atkins et al. 1999).

***Armillaria* species are diverse in pathogenicity, host-specificity, and general environmental requirements.** *Armillaria* species are prominent killers and beneficial decayers of deciduous and coniferous trees and shrubs in natural forest, plantation, orchard, agroforestry, and urban

settings (Shaw and Kile 1991). To date, ten biological species of *Armillaria* are known on the North American continent (Anderson and Ullrich 1979, Banik and Burdsall 1998, Banik et al. 1996, Guillaumin et al. 1991, Volk et al. 1996). These fungi are often referred as North American Biological Species (i.e., NABS) with a Roman numeral designation (Table 1) (Anderson 1986). They are diverse in pathogenicity, host-specificity, and general environmental requirements (Table 1). For example, *A. ostoyae* and *A. mellea* are prominent killers of various coniferous and deciduous trees and shrubs. In the Pacific Northwest, Douglas-fir and grand fir are generally among the species that are most susceptible to Armillaria root rot, whereas western white pine (*Pinus monticola*), western larch (*Larix occidentalis*), and ponderosa pine (*Pinus ponderosa*) are typically less susceptible. However, susceptibility to Armillaria disease varies with the site conditions, physiological status of the tree, and pathogen isolate. In the wet inland forests of northern Idaho, eastern Washington, and western Montana, *Armillaria* can cause severe mortality in both Douglas-fir and grand fir in size classes ranging from seedlings to mature trees. In higher elevation forests, *Armillaria* causes damage to subalpine fir (*Abies lasiocarpa*) and Engelmann spruce (*Picea engelmanii*) (McDonald 1999). The fungus is seldom found in dry forests dominated by ponderosa pine (McDonald 1999).

Table 1. *Armillaria* species and relative pathogenicity

NABS [a]	Species	Pathogenicity[b]	Primary Host	Range[c]
I	*A. ostoyae*	High	Conifer	Northern conifer zone
II	*A. gemina*	Unknown	Unknown	Northeastern U.S.A., Québec, Ontario
III	*A. calvescens*	Low?	Unknown	Québec to Michigan and Wisconsin, Canadian prairie
V (IV)	*A. sinapina*	Low?	Mixed	Northern conifer zone, but usually on hardwoods in the East and conifers in the West
VI (VIII)	*A. mellea*	High	Hardwood	Mostly southeastern U.S.A., north to Iowa and Wisconsin and west to Oklahoma and Texas. Also known from California, but not other areas of the West.
VII	*A. gallica*	Low?	Hardwood	Southeast to Northeast and Midwest, rare in Pacific Northwest
IX	*A. nabsnona*	Unknown	Hardwood?	Known from Idaho, Washington, Oregon, Alaska, and British Columbia
X	*Unnamed*	Low?	Mixed	Known only from British Columbia and Idaho
XI	*A. cepistipes*	Low?	Mixed	Known from British Columbia and Washington
	A. tabescens		Mixed	Southeastern U.S.A. into the Northeast, west to Ohio, also further west and north to Great Lakes

[a]NABS = North American Biological Species
[b]Pathogenicity of some *Armillaria* species is largely unknown.
[c]From http://www.wisc.edu/botany/fungi.arm.html

Can beneficial *Armillaria* species provide protection from infection by pathogenic *Armillaria* species? Some *Armillaria* species (e.g., *A. sinapina*, *A. gallica,* and NABS X) are predominately saprophytic, and should improve forest productivity by improving nutrient cycling through decomposition of organic matter. Recent studies also indicate that such *Armillaria* species may protect susceptible trees from attack by pathogenic *Armillaria* spp. (Bruhn et al. 2000). Surveys indicate pathogenic and saprophytic *Armillaria* species occur in close proximity in the forests of northern Idaho (McDonald et al. 1998a). The role of saprophytic *Armillaria* species in maintaining forest health warrants further investigation. Because management of Armillaria root disease over extensive areas is seldom practical, management practices that favor beneficial saprophytic *Armillaria* species adapted to local conditions may offer an attractive alternative for managing Armillaria root disease if conditions could be identified.

Molecular genetics offers new methods to augment species identification, determine species and population distributions, and generate markers for assessing genetic structure and ecological behavior of individual species and populations. Because *Armillaria* species can have similar morphologies in culture, isolates from trees or forest soils must be reliably identified before impacts on production, either negative or positive, can be assessed or predicted. In previous studies, methods were developed to identify species on the basis of reactions between paired isolates in culture (Guillaumin et al. 1991, Harrington et al. 1992, Mallet et al. 1989, McDonald et al. 1998b, Harrington and Rizzo 1993, Rizzo et al. 1995). In addition, molecular genetic methods have been used to verify species identifications (Anderson and Stasovski 1992, Harrington and Wingfield 1995, Kim et al. 2000, Kim et al. 2001, Miller et al. 1994, Schulze et al. 1995, 1997, Smith and Anderson 1989, Smith et al 1990). Compared to conventional culture methods, molecular-based methods are simple, low cost, reliable, and fast. These identification methods are fundamental to the successful study and management of forests influenced by pathogenic and nonpathogenic *Armillaria* species.

Methods such as Random Amplified Polymorphic DNAs (RAPD), Amplified Fragment Length Polymorphisms (AFLP), and Random Amplified Microsatellites (RAMS) can generate abundant genetic markers whose presence or absence can be assessed for correlations with local environment, influences on tree growth, phylogenetic relationships, and gene flow within and among microbial populations (Bakkeren et al. 2000, Hogberg et al. 1999, Pei and Ruiz 2000, Wetzel et al. 1999).

AFLP is a powerful technique for "fingerprinting" genomic DNA. DNA "fingerprinting" is used to visualize large numbers of DNA polymorphisms between samples. Genetic markers used for fingerprinting can also be used to generate linkage maps or to identify genetic markers linked to phenotypic traits and/or genetic loci. AFLP markers can be obtained from *Armillaria* pedigrees bearing potentially any physiological trait of interest (e.g., pathogenicity, site adaptability, substrate utilization, antibiotic production, mating, vegetative compatibility, or capacity for bioremediation). This method can also be used to identify species, populations, and genets of *Armillaria* and assess gene flow among *Armillaria* populations. In theory, such genetic markers can be correlated with environmental factors associated with diverse *Armillaria* species and populations occupying a diverse spectrum of habitats.

Basic research questions

- Will AFLPs discriminate among *Armillaria* species, populations, and genets (vegetative clones)?
- Can pathogenic vs. nonpathogenic *Armillaria* species and populations be distinguished using AFLP markers?
- Can *Armillaria* populations be identified that possess unique host ranges or defined environmental requirements?
- Do habitat type, elevation, slope, aspect, etc. affect spatial geographic patterns within *Armillaria* species or populations?
- What is the relationship among the occurrences of different *Armillaria* species/populations?
- To what extent can we correlate the occurrence of specific genetic markers within *Armillaria* species and populations with specific environments (e.g., sites, host, pathogenicity, and environmental factors)?
- Do certain environments foster greater genetic diversity via gene flow?
- Can the occurrence of different *Armillaria* species and their associated ecological behaviors (e.g., host range and pathogenicity) be predicted for specific locations?

Objectives

1. Develop genetic marker systems for identifying species and distinguishing populations and genets of pathogenic and nonpathogenic *Armillaria* species
2. Determine what significant correlations exist among species (or identifiable populations within species), host preference, and other environmental variables (e.g., habitat type, geographic location, elevation, slope, aspect, etc.)
3. Use genetic markers to predict the ecological behavior and occurrence of *Armillaria* species and populations, and
4. Begin development of methods to integrate predictions of *Armillaria* behavior into a landscape model for use in forest ecosystem management.

RATIONALE AND SIGNIFICANCE

The greatest significance of this project is its novel approach relating the genetic structure of *Armillaria* species and populations with environmental factors (and ecological function) at the landscape level. This fundamental knowledge is needed to facilitate prediction and management of beneficial and pathogenic *Armillaria* species to improve sustainability and productivity of forest ecosystems in the United States. About 10% (28 million ha) of forested land is currently at risk (where 25% or more tree mortality is expected over the next 15 years) and a substantial proportion of this mortality is due to *Armillaria* spp. (USDA Forest Service FHP 1999). Human demands on natural and managed forests for fiber, energy, and other benefits (e.g., recreation, watersheds, and wildlife habitat) are significantly increasing. However, benefits derived from forests are increasingly limited by biotic and abiotic disturbances. The magnitude of direct and indirect benefits from healthy forests is clearly larger than those available in forests suffering high mortality. Therefore, the maintenance and sustainability of forest health is of paramount

importance, and management of Armillaria disease must be a critical component of any management strategy. It is reasonable to expect that the integration of diagnostic genetic markers with critical environmental factors at the landscape level will promote the development of novel prediction and management methods for Armillaria root disease. In addition, this proposed study provides a feasible approach that can be applied to extensive forest ecosystems: Pathogens cannot be manipulated by chemicals in natural systems (as is common in other agricultural systems), but populations of pathogenic and beneficial microbes can be subtly managed by environmental conditions fostered by vegetation management and other silvicultural practices. The concepts and innovations developed in the proposed study will also be instrumental toward understanding pathogen ecology and developing prediction and management systems for various pathogens in diverse environments.

PRELIMINARY STUDIES

A total of 3,963 *Armillaria* isolates have been collected from study plots in northern Idaho. The collection site was located in USDA Forest Service, Priest River Experimental Forest (PREF), Bonner County, Idaho. The following information is a brief description of sampling methods and *Armillaria* species/genets identification.

Sampling Methods - Three regions (each 12.1 ha) were delineated within PREF (2,800 ha) that corresponded to low (Region 1; > 890 m), middle (Region 2; 890 – 1,005 m), and high (Region 3; <1,005 m) elevation (Fig. 1). Six blocks (replications) were then established across each region and total of 20, 0.04-ha plots were installed on each block (Fig. 1.). Thus, each elevational region contained 120 plots, for a total of 360 plots across three elevational regions. The habitat type of each plot was determined and recorded according to Cooper et al. (1991). The five most common sub-series[1)] (groups of habitat type) (see McDonald et al. 2000) for these regions are *Cool fir/Dry shrub*, *Cool fir/Moist herb*, *Cedar Hemlock/Dry shrub*, *Cedar Hemlock/Moist herb*, and *Dry fir/Dry shrub* (Fig. 1). Within each plot, three individuals (three different diameter classes) for every tree and hardwood shrub species present were surveyed for the occurrence of *Armillaria* (rhizomorphs, mycelial fans, or wood decay) and pathogenic colonization by *Armillaria* (e.g., resinosis) (Worrall and Harrington 1988).

Isolations and Species/Genets Identification - Rhizomorphs, mycelial fans, or wood decay were collected from surveyed trees and shrubs, surface-disinfested, and established in culture. These plots yielded 910 *Armillaria* isolates, which were condensed to 46 genets (designated "Group A") by somatic paring (Guillaumin et al. 1996, Harrington et al. 1992, Mallet et al. 1989, Rizzo et al. 1995). Based on somatic pairing, haploid mating (Anderson and Ullrich, 1979, Anderson 1986, Banik and Burdsall 1998, Banik et al. 1996, Guillaumin et al. 1991, Volk et al. 1996), IGS restriction length polymorphisms (Harrington and Wingfield 1995, Kim et al. 2000, Kim et al. 2001) and IGS sequence analysis (Anderson and Stasovski 1992, Coetzee et al. 2000), these 46

[1)] *Cool fir*: *Abies grandis* (grand-fir); *Dry fir*: *Pseudotsuga menziesii* (Douglas-fir); *Cedar Hemlock*: *Thuja plicata* (western red cedar), *Tsuga heterophylla* (western hemlock); *Dry shrub*: *Physocarpus malvaceus* (ninebark); *Moist herb*: *Clintonia uniflora* (queen cup beadlily)

genets were comprised of *A. ostoyae*, *A. sinapina, A. gallica*, and NABS X. In addition, 80 more *Armillaria* genets ("Group B"; derived from 3,053 *Armillaria* isolates) are also available for this project. These genets were collected from plots randomly scattered throughout PREF. These plots are representative of sub-series[2)] that are present within PREF. The same sampling methods as described above were applied for isolating *Armillaria*. Habitat type, pathogenic colonization by *Armillaria*, and other associated information (host species, habitat type, health status and size of host, elevation, slope, and aspect) of these genets have been documented for each collection, but their taxonomic identification determined by haploid pairing has not been verified by IGS sequencing.

Figure 1. Potential vegetation sub-series (groups of habitat types) on the Priest River Experimental Forest as mapped by most similar neighbor (MSN) imputation method (McDonald et al. submitted, Moeur and Stage 1995). The PREF stands classified to eight sub-series. Lower right box shows Region 2 (middle elevation; 890 - 1,005 m) plot location. Each block within a region contained 20 plots. Each colored dot (red, blue, green, beige, and dark blue) represents different sub-series.

Figure 1 is located on the next page

Highly variable AFLP genetic markers have been obtained from *Armillaria* genets. We screened 16 selective primer combinations using six *Armillaria* genets (three from *A. sinapina* and three from NABS X). Among 16 selective primer sets, we chose 3 that produced the most manageable band number and interpretable banding pattern. AFLP markers from these three primer pairs visualized on an ABI 377 DNA sequencer (Applied Biosystems, Inc., Cambridge, MA) and scored using Genotyper software (Applied Biosystems, Inc., Cambridge, MA) are summarized in Tables 2 and 3. An example of AFLP fingerprinting from amplification with E-AC/M-CAA of DNA from three NABS X genets is shown in Figure 2. This preliminary study showed that the AFLP technique can generate useful markers to discriminate *Armillaria* genets.

Table 2. Polymorphisms revealed using AFLP for genet discrimination among three different genets of *A. sinapina*.

Primer pair	Number of bands	Number of polymorphic bands among three genets of *A. sinapina*	% polymorphic bands among three genets of *A. sinapina*
E-AC/M-CAA	55	12	22%
E-AC/M-CTG	51	11	22%
E-AC/M-CTT	59	12	20%

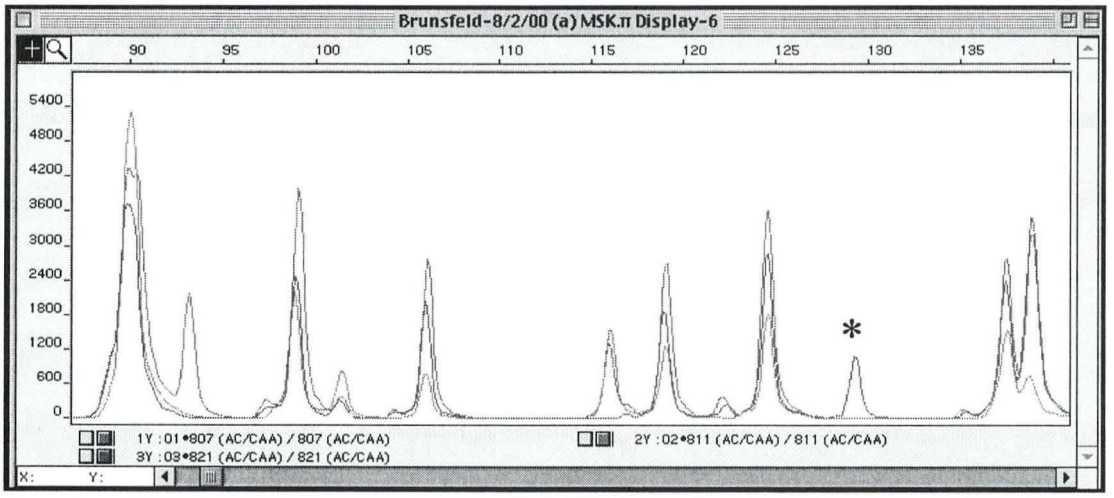
[2)] *Cold fir/Dry herb*, *Cold fir/Moist herb*, *Cedar Hemlock/Wet fern*, and *Cedar Hemlock/Wet Herb*
Cold fir: *Abies lasiocarpa* (subalpine fir); Dry herb: *Linnaea borealis* (twinflower); Wet fern: *Athyrium filix-femina* (ladyfern); Wet herb: *Viola glabella* (pioneer violet), *Gymnocarpium dryopteris* (oak fern), *Asarum caudatum* (wild ginger)

Table 3. Polymorphisms revealed using AFLP for genet discrimination among three different genets within NABS[a] X.

Primer pair	Number of bands	Number of polymorphic bands among three genets of NABS X	% polymorphic bands among three genets of NABS X
E-AC/M-CAA	47	13	28%
E-AC/M-CTG	60	14	23%
E-AC/M-CTT	63	11	17%

[a] NABS = North American Biological Species

Figure 2. An example of AFLP fingerprinting of *Armillaria* NABS X. Blue, green, and red colors represent different individuals (genets) of NABS X



The X axis represents band size (bp) and the Y axis represents band intensity. An example of a polymorphic band is denoted with an asterisk (*). Genet 821 (red) has a 129-bp band, whereas genets 807 and 811 (blue and green) do not have the 129-bp band. Note: Color image is required for optimal interpretation.

RESEARCH METHODS

The study has five primary experimental phases:

1. Taxonomic identification of remaining 80 "Group B" *Armillaria* genets from PREF using DNA sequencing (intergenic spacer region).
2. Verification of *Armillaria* genets identified from somatic pairing test using AFLP.
3. Generation of genetic markers for all *Armillaria* genets using AFLP.
4. Statistical analysis to discriminate species and populations, and analyze relationships of host species, habitat types, geographic locations, elevation, slope, and aspect with spatial distribution.
5. Develop information for use in predictive models for *Armillaria* root disease.

Rationale for approach. Whereas, the DNA sequencing method is one of the most reliable techniques to identify *Armillaria* species, AFLP represents a very powerful and reliable method to generate 1,000's of genetic markers. Both technologies are already available and successfully tested with *Armillaria* species (as mentioned above). Priest River Experimental Forest (PREF) is an ideal site to conduct the proposed study. It has very complete historical records going back nearly 100 years, and vegetation and management history have already been mapped. The USDA Forest Service, Forest Sciences Laboratory (Moscow, Idaho) has 126 well-characterized genets of *Armillaria* collected from PREF. These 126 genets (46 from Group A and 80 from Group B) are the core materials for this proposed study. These genets and their related biological information represent 15 years of *Armillaria* research in the PREF.

The experimental procedures for research tasks 1-5 (above) are described in the following.

1. Identification of *Armillaria* species using DNA sequencing: The intergenic spacer (IGS) region of ribosomal gene will be amplified and sequenced for species identification by phylogenic comparison with known *Armillaria* species (Coetzee et al. 2000). Species identification for Group A (total 46 genets) was completed using IGS sequencing, IGS-RFLP, and somatic/haploid pairings. Taxonomic identification will also be performed for Group B (total 80 genets). Template DNA for IGS amplification will be derived from mycelial scraping, so no DNA extraction is necessary in this step. Sequencing will be performed using an ABI 377XL PRISM automated sequencer (Applied Biosystems, Inc., Cambridge, MA) and fluorescently labeled di-deoxy terminators. Sequences will be assembled with Sequencher 3.1 (Gene Codes Corporation, Ann Arbor, MI) and phylogenetic analysis will be conducted using PAUP (Sinauer Associations, Inc., Publishers, sunderland, MA), Phylip, or other appropriate softwares. Based on previous pairing test, we expect that four or five *Armillaria* species (*A. ostoyae*, *A. sinapina*, *A. gallica*, *A. nabsnona*, and NABS X) will be identified from this area (McDonald et al. 1998a) of which *A. ostoyae* is a pathogenic species that kills trees, while the other species (*A. sinapina, A. gallica, A. nabsnona,* and NABS X) are predominately saprophytic.

2. Verification of *Armillaria* genets identified from somatic pairing test using AFLP: A total of 12 genets (three from each of *A. ostoyae*, *A. sinapina*, *A. nabsnona*, and NABS X) from Group A and B will be used for verifying the somatic pairing method previously used for genet identification. Five to ten isolates (depends on availability) from each genet will be randomly selected for this experiment. For the generation of AFLPs, 250 ng of *Armillaria* genomic DNA will be digested with *Eco*R I and *Mse* I to serve as the template. Resulting DNA fragments will be ligated to adapters and preamplified with polymerase chain reaction (PCR). Fluorescent dye-labeled, *Eco*R I selective primers will be used for the final PCR amplification. Products will be separated on polyacrylamide gel electrophoresis using an ABI 377 DNA sequencer (Applied Biosystems, Inc., Cambridge, MA). In addition, we will test and screen more selective primer sets to generate higher quantity and quality of markers for subsequent use. Eventually, we will apply about 3-4 selective primer sets per isolate to compare band profiles among isolates within each genet. AFLP markers will be analyzed using Genotyper C to create binomial data sets (present [1] or absent [0]). AFLP data sets will be transferred to a spreadsheet for genetic and statistical analyses. Several genetic and statistical analyses (e.g., a genetic similarity, genetic diversity, cluster analysis, non-metric multidimensional scaling or principal coordinate analysis,

etc.) can be performed to verify genets that were previously identified by the somatic pairing method (McDonald 1998b). Detailed genetic and statistical analyses will be explained in Research task #4 below.

3. Generation of genetic markers from each *Armillaria* genets: In our preliminary study, an average of ca. 56 bands per selective primer set was generated for each *Armillaria* genet. We will apply about 3-4 selective primer sets (resulting from additional screening process; research task #2) per genet in our proposed study. Therefore, about 200 markers (3-4 primers * ca. 56 or more bands) will be generated from each genet. A total of ca. 25,000 (ca. 200 markers * 126 genets) markers will be analyzed to correlate with environmental factors (host species, habitat types, elevation, slope, aspect, etc.) The binomial AFLP data sets (present [1] or absent [0]) will be transferred to a spreadsheet for genetic and statistical analyses.

4. Statistical and spatial analyses:
Statistical analysis: The binomial data will be used to calculate genetic similarities (e.g., Dice's similarity coefficients) for all possible pairwise comparisons (126 X 126) of individuals ("Group A & Group B" genets). Based on genetic similarity value, associations among genets will be revealed by a cluster analysis with the unweighted pair-group method using arithmetric average (UPGMA; NTSYS-pc software package; Rohlf 1992) or other appropriate methods. Second, non-metric multidimensional scaling (MDS) method will be applied to describe which environmental parameters (e.g., host species, habitat type, geographic location, etc.) account for genet clustering. Principle coordinate analysis also can be applied. Genets may be clustered by host species, habitat type, occurrence of disease, elevation, slope, aspect, and geographic location (distance).

The logistic multiple regression (LMR) technique will be applied to identify variables important in predicting the probability of marker occurrence (1 or 0). Quantitatively, the relationship between the "occurrence" (1 or 0) and its dependency on several variables (habitat type, host species, pathogenic colonization, elevation, slope, and aspect, etc.) can be expressed as:

$$\text{Log}\,[d] = b_0 + b_1x_1 + b_2x_2 + \ldots b_px_p$$

where d = [Prob (present marker)/Prob (absent marker)]

$x_1 \ldots x_p$ = a set of environmental data (host species, habitat type, elevation, slope, aspect, geographic location - distance)

$b_1 \ldots b_p$ = coefficients derived from logit regression

Expressed in simpler terms, d is the dependent variable (nominal scale, 1 or 0 in this study) and, $x_1 \ldots x_p$ are independent variables (environmental data). The binary response variable data layer could be used to investigate the relationship between response probability and the explanatory variables. Thus, the logistic multiple regression will be ideal for developing a predictive model for this study. More detailed statistical analysis will be modified to reflect the needs of this study. All statistical analyses will be performed with the appropriate statistical software packages.

Spatial analysis. These studies will be performed by collaborator McDonald. One of the primary sub-objectives of this work is to evaluate the spatial context in which specific *Armillaria* species and/or genetic markers occur. In addition to the regression analysis described above, which will

quantify the relationship between the occurrence of specific markers and environmental factors (mainly habitat types) at the study sites, GIS technology will be used to visualize and evaluate the occurrence of markers across the landscape in combination with landscape distribution of habitat types and other environmental parameters (e.g., host species, elevation, slope, aspect, and geographical locations).

This analysis consists of two steps. The first step will be to qualitatively evaluate these spatial relationships with a series of overlays within the GIS. The various species and markers observed at the field plots will be overlayed with the spatially distributed landscape factors (e.g., habitat type, elevation, slope, and aspect). The second step in this analysis will be to quantify of any qualitative relationships visualized in the first step.

5. Development of predictive models for Armillaria root disease based on habitat types:
If the statistical analysis with the field plot data yields significant relationships between any habitat type, host species, elevation, slope, or aspect and *Armillaria* distribution or behavior, then the derived regression relationships can be used to map the predicted distribution or behavior of *Armillaria* across the landscape. Based on previous studies, the genus *Armillaria* is expected to be widely distributed (e.g., continuous) across the PREF study sites (McDonald unpublished). It should be clarified that "prediction" here refers to spatially mapping those areas most prone to occurrences of particular species and markers. Significant relationships uncovered in the analysis of spatial patterns of *Armillaria* occurrence and environmental factors will be incorporated into the predictive mapping strategy. Again, the techniques to be used will depend on the nature of the spatial relationships.

Application of results

This study will provide critical information on the influence of habitat type (and associated environmental factors such as host species, elevation, slope, and aspect) on the distribution, genetic structure, and ecological behavior of *Armillaria* species and populations at the landscape level. Information on ecological relationships of pathogenic and beneficial *Armillaria* spp. will facilitate the development of models to predict *Armillaria* distribution across the forest landscapes. Such information can be also applied to determine appropriate management practices (e.g., species selection, thinning and harvest methods, site preparation, etc.) for specific forest sites, so that beneficial effects of saprophytic *Armillaria* spp. are increased and detrimental effects of pathogenic *Armillaria* spp. are minimized. Such approaches offer a unique opportunity to manage Armillaria root and butt rot disease, and help maintain healthy and sustainable forest resources. This study also lays a strong foundation for examining the environmental influences on pathogen genetic structure and distribution for other pathosystems by developing novel methods to integrate molecular genetics of pathogens with GIS and environmental data at the landscape level.

Limitations and Pitfalls

I comment on several issues that have concerned me and my collaborators while writing this proposal, and thus may also be of concern to reviewers.

- Does the PI have sufficient experience with spatial analysis and GIS-based mapping? I will closely collaborate with Dr. McDonald (USDA Forest Service) and GIS specialists, Tom Rice and Jeffrey Evans (USDA Forest Service), who are currently engaged in mapping *Armillaria* distribution (letter of collaboration) from habitat typing. I will share co-authorship of papers and presentations from this proposed study with these scientists.

- Can the results of this proposed study be projected to other ecological regions of United States? It is very difficult to predict whether results from this proposed study can be readily applied in the southeastern United States due to a totally different *Armillaria* species composition and differing environments. However, establishing the relationship between environmental factors and *Armillaria* distribution is critical to our understanding of this pathosystem. The proposed framework for interpreting interactions at the landscape level is applicable to widely ranging pathogens and environments. However, the approach in this proposal will provide fundamental guidelines to predict *Armillaria* behavior and manage impacts of beneficial and pathogenic *Armillaria* spp.

- Would sampling *Armillaria* isolates from diverse environments across the northwestern United States provide more information than samples collected from the relatively small area of our proposed study site (PREF, 2,800 ha)? A focused approach is necessary to provide the resolution needed for understanding ecological interactions of *Armillaria* spp. Environments within our study site are well-characterized and are quite diverse. After the ecological relationships of *Armillaria* spp. are understood at a practical scale, these studies can be applied to on a broader geographic scale.

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